Indian J. Genet., 81(1): 43-49 (2021)
DOI: 10.31742/IJGPB.81.1.4

Molecular tagging of *Rf* genes for the fertility restoration of WA-CMS system by bulk segregant analysis in rice

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(Received: June 2020; Revised: December 2020; Accepted: January 2021)

Abstract

The process of screening for fertility restoration trait involves test crossing with a set of cytoplasmic male sterile (CMS) lines and evaluation of F_1 hybrids for pollen and spikelet fertility. In the present study, F_2 mapping population derived from a cross, APMS 6A × RP 5933-123 was utilized to map *Rf* genes. The F_2 population was also genetically analysed for pollen and spikelet fertility percentage. Chi-square ($\chi 2$) analysis to showed that the fertility restoration trait followed expected digenic ratio. By bulk segregant analysis (BSA) likely *Rf* genes containing regions were located on chromosome 10. The SSR markers *viz*., RM304, RM258 located on chromosome 10 and RM23958 located on chromosome 9 showed clear polymorphism between two groups of fertile and sterile bulks. Based on BSA linkage analysis and F_2 population, pollen and spikelet fertility analysis along with molecular screening results of *Rf* linked markers, it is concluded that *Rf4* gene located on chromosome 10 is playing major role and contributing to 90% of fertility restoration trait of newly derived restorer line RP5933 along with minor effect genes from chromosome 9. The findings may be useful for rice hybrid breeding.

Keywords: Hybrid rice, *Rf genes*, WA-CMS system, BSA, Fertility restoration

Introduction:

Rice *(Oryza sativa* L.) is one of the most important food crops for half of the world's population. The world rice production is 503 million tonnes harvested from 162 m.ha. during 2019, whereas in India rice is cultivated in an area of 43.7 million hectares with the production of 118 m.t. (FAOSTAT 2019). The current production has been possible through the release and cultivation of high yielding varieties and hybrids with good agronomic practices. China produces 142 m.t. of rice from 29.5 m.ha. itself, by cultivating hybrid rice in an area of 60% of the total rice cultivated area (http://ricestat.irri.org). In India with limited resources like land, water, labour and other inputs, rice productivity needs to be increased without disturbing the environment. Hybrid rice technology is the pragmatic approach to increase rice production of 15 to 20 per cent than the pure line varieties by exploiting hybdrid vigour.

Use of cytoplasmic genetic male sterility/fertility restoration (CGMS/*Rf*) system is a potential approach for hybrid rice seed production. In CGMS hybrid rice seed production involves three lines viz., cytoplasmic male sterile (CMS) line (A), a maintainer line (B) and a restorer line (R). Even though several independent CMS sources (more than 20) have been reported, of which only three namely, *viz*., Wild Abortive (WA), Boro Tai (BT) and Honglian (HL) are extensively employed for commercial hybrid seed production (Fujii and Toriyama 2005). Of these, the WA system is widely used CMS source for *indica* rice accounting for about 90% of the rice hybrids produced in China and 100 % of the hybrids developed outside China (Sattari et al. 2007). For developing high yielding heterotic hybrids, the first step is to identify potential restorers that can efficiently restore the fertility of F_1. The process of screening for fertility restoration is laborious and time-consuming as it involves test crossing with a set of CMS lines and evaluation of resultent F_1s for pollen and spikelet fertility. The use of molecular markers linked to *Rf* genes can enhance the selection efficiency, save time and avoid the complications associated with phenotype-

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Published by the Indian Society of Genetics & Plant Breeding, A-Block, F2, First Floor, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org; indianjournals.com

based screening (Revathi et al. 2013). Markers linked to *Rf* genes can be utilized to accelerate the process of breeding restorer lines for developing rice hybrids (Rashid et al. 2019).

Studies on genetic analysis of fertility restoration for predominantly used WA-CMS lines indicated that two major genes Rf_3 and Rf_4 located on chromosomes 1 and 10, respectively are responsible for restoring the fertility (Yao et al. 1997; Zhang et al. 1997; Tan et al. 1998). Subsequent studies using molecular markers indicated the involvement of more than two genes/ QTLs governing fertility in WA-CMS lines (Zhuang et al. 2000). The WA-CMS fertility is restored by the dominant and sporophytic restorer genes (*Rf*) (Li and Yuan 1986; Virmani 1996). The various studies on inheritance of fertility restoration trait demonstrated that fertility restoration is governed a single (Wang 1980; Shen et al. 1996), two linked genes (Li et al. 1982), two independent genes (Li and Yuan 1986; Virmani et al. 1986; Govindaraj and Virmani 1988; Bharaj et al. 1991; Teng and Shen 1994), and polygenes (Pei 1980). Furthermore, seven chromosomal locations for such genes have been reported: one on chromosome 1 (Zhang et al. 1997), two on chromosomes 7 and 10 (Bharaj et al. 1995) and four QTLs on chromosomes 2, 3, 4, and 5 (Zhu et al. 1996).

Molecular mapping of the *Rf* genes provides a powerful tool to develop restorer lines avoiding extensive test crossing with CMS lines (Hu et al. 2016). Molecular tagging of the fertility restorer gene will help in its transfer to other desirable agronomic background without involving a sterile cytoplasm or extensive test crossing with CMS lines. Ultimately, the identification of closely linked markers would help in map based cloning of the fertility restorer gene to understand the molecular and biochemical basis of fertility restoration and the possible role of fertility restoring gene (s) in other biological processes (Mishra et al. 2003). The bulk segregant analysis (BSA) and selective genotyping are quick and reliable methods used to tag molecular markers linked to a particular trait in segregating mapping population (Collard et al. 2005). BSA has been successfully deployed in rice and other cereals for identifying markers linked to several traits viz., grain yield, drought tolerance, salinity tolerance, disease resistance, heat tolerance and quality parameters (Raveendran and Valarmathi 2020). The present investigation has been carried out to study the inheritance of fertility restoration and tag new fertility restorer *Rf* gene(s) using BSA in the F_2 population derived from the cross APMS 6A and a newly developed restorer line RP5933-123, which showed absence of *Rf4* and *Rf3* genes by molecular screening.

Materials and methods

The plant material consisted of WA-CMS line APMS 6A which is a female parent of popular rice hybrid DRRH 3 and a newly developed restorer line RP-5933-123 developed at Indian Institute of Rice Research (IIRR), Hyderabad. By crossing APMS 6A with RP-5933-123, F_1s were produced and true F_1 plants were selfed to generate F_2 population. The segregating F_2 population was grown under standard crop management practices at IIRR, Rajendranagar, Hyderabad during *kharif* 2016. The phenotypic observation of pollen and spikelet fertility was recorded in each F_2 individual as per standard procedure.

Pollen and spikelet fertility

Pollen fertility percentage was calculated using anthers collected from spikelets at 1 to 2 days before anthesis. The anthers from each spikelet were smeared in a drop of 1% Iodine-potassium iodide (I-KI) solution on a glass slide and three randomly selected microscopic fields were counted. Stained, well filled and round pollen grains were counted as fertile, while unstained, shriveled and empty pollen grains were considered as sterile. Pollen fertility was calculated and expressed in percentage as a ratio of a number of stained pollen grains to the total number of pollen grains examined. Based on the pollen fertility percentage the pollen parent was classified as fertile (60-100%), partial fertile (30-59.99%), partial sterile (1-29.99%) and sterile (0-0.99%) according to Virmani et al. (1997).

Spikelet fertility percentage was calculated by bagging the panicles that emerged from the primary tiller before anthesis to avoid out crossing. The number of filled grains and chaffs in the panicle were counted at the time of maturity. The ratio of filled grains to the total number of spikelets was expressed as spikelet fertility and expressed in percentage as a ratio of a number of filled spikelets per panicle to the total number of spikelets in the panicle. Based on the spikelet fertility percentage genotypes were classified as fertile (75-100%), partial fertile (50-74.9%), partial sterile (1-49.9%) and sterile (0%). The pollen and spikelet fertility data were subjected to chi-square analysis to test the goodness of fit Virmani et al. (1997).

Genotyping and Bulk Segregant Analysis (BSA)

A total of 414 simple sequence repeat (SSR) markers

spread across 12 rice chromosomes were utilized for parental polymorphism survey between APMS 6A and RP 5933-123. The total genomic DNA was extracted from 30 days old leaves as per protocol reported by Dellaporta et al. (1983). Polymerase chain reaction (PCR) was performed in a thermal cycler (Eppendorf, Germany) using a total reaction volume of 10 µl. The reaction mixture contained 30 ng of template DNA, 5 pmol of each of primer, 2.5 mM dNTPs, 10x PCR buffer and 1 unit of Taq DNA polymerase. The polymerase chain reaction comprised of one cycle of denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension of 72°C for 7 min. The amplified products were resolved on 3% SeaKem LE Agarose (Lonza USA) gel containing 0.1 mg/ml of ethidium bromide along with 100 bp DNA ladder and visualized on ultraviolet transilluminator and results were documented.

The identified polymorphic SSR markers between parents were utilized for bulk segregant analysis (BSA). The fertile and sterile bulks were formed by mixing DNA isolated from corresponding fertile (pollen and spikelet fertility > 90%) and sterile (0 % pollen and spikelet fertility) F_2 plants and BSA analysis was performed as described by Tan et al. 1998; and Ahmadikhah et al. 2007. The recessive class anlaysis was carried out to estimate recombination frequency for linkage mapping as explained by to Zhang et al. (1994).

Results and discussion

The indicated pollen and spikelet fertility percentage in F_1 that RP 5933-123 is a complete restorer with more than 90% of pollen and spikelet fertility. The results of molecular screening of restorer line, RP 5933-123 with RM6100 and DRRM-Rf3-10 SSR markers linked to *Rf4* and *Rf3* genes indicated the absence of both the genes. The 250 F_2 plants derived from a F_1 (APMS 6A x RP5933-123) were analysed for pollen and spikelet fertility percentage to study the inheritance of fertility restoration. These F_2 plants were classified into fertile, partial fertile, partial sterile and sterile based on their pollen and spikelet fertility percentage according to Virmani et al. (1997). Assuming that fertility restoration was governed by two major genes the chi-square (χ^2) test was carried out, to test for the goodness of fit of the P values. The results are presented in Tables 2 and 3. The frequency of classified plants fitted well to the expected digenic ratio indicating the presence of two major fertility restorer genes in the restorer RP 5933-123. Digenic fertility restoration for WA-CMS system has been reported earlier by several researchers (Govindaraj and Virmani 1988; Bharaj et al. 1991; Yao et al. 1997; Hossain et al. 2010). The table value of (χ^2) at 0.05 level of probability at 3 df was 7.82. Since calculated (χ^2) values for pollen and spikelet fertility percentage were lower than that of table value of (χ^2) explaining that fertility restoration trait is governed by two dominant independent genes. The present study also found fertility restoration of RP 5933-123 restorer is also governed by two dominant independent *Rf* genes.

Identification of polymorphic markers and Bulk Segregant Analysis

A genome-wide parental polymorphism survey was carried out between parents (APMS 6A and RP5933-123) using a total of 414 SSR markers spanning 12 rice chromosomes (http://www.gramene.org/) and 40 SSR markers were found polymorphic between APMS 6A and RP5933-123 (Table 1). The average per cent polymorphism between parents was 10.14% only. Molecular markers have enabled researchers to precisely map *Rf* genes for WA-CMS system. Identification of linkage between target genes and molecular markers can be achieved by developing different mapping populations like F_2, recombinant inbred lines (RILs), near-isogenic lines (NILs) and backcross generations etc. Bulk segregant analysis using F_2 mapping population is very effective in identifying DNA markers associated with the target genes in a short period. Use of BSA in mapping *Rf* genes for WA-CMS system has been demonstrated by Tan et al. (1998) and Ahmadikhah et al. (2007).

In the present study, F_2 individuals derived from the cross APMS 6A X RP 5933-123, ten plants each showing complete fertility (>90 % of pollen and spikelet fertility) and ten plants showing complete sterility (0% of pollen and spikelet fertility) were selected and DNA was isolated and PCR analysis was carried out with the help of identified 40 polymorphic markers between parents (Table 1). In BSA, SSR markers *viz*., RM304, RM258, located on chromosome 10 and RM23958 located on chromosome 9 showed polymorphism between fertile and sterile bulks among the 40 tested SSR makers (Fig. 1). Gyan et al. (2002) reported that RM258 is linked to fertility restorer gene at a distance of 9.5 cM in the basmati restorer line PRR-78. He et al. (2002) demonstrated that RM1, RM258, and RM304 are linked to fertility restorer genes at a distance 1.9, 2.9 and 0.0 cM, respectively.

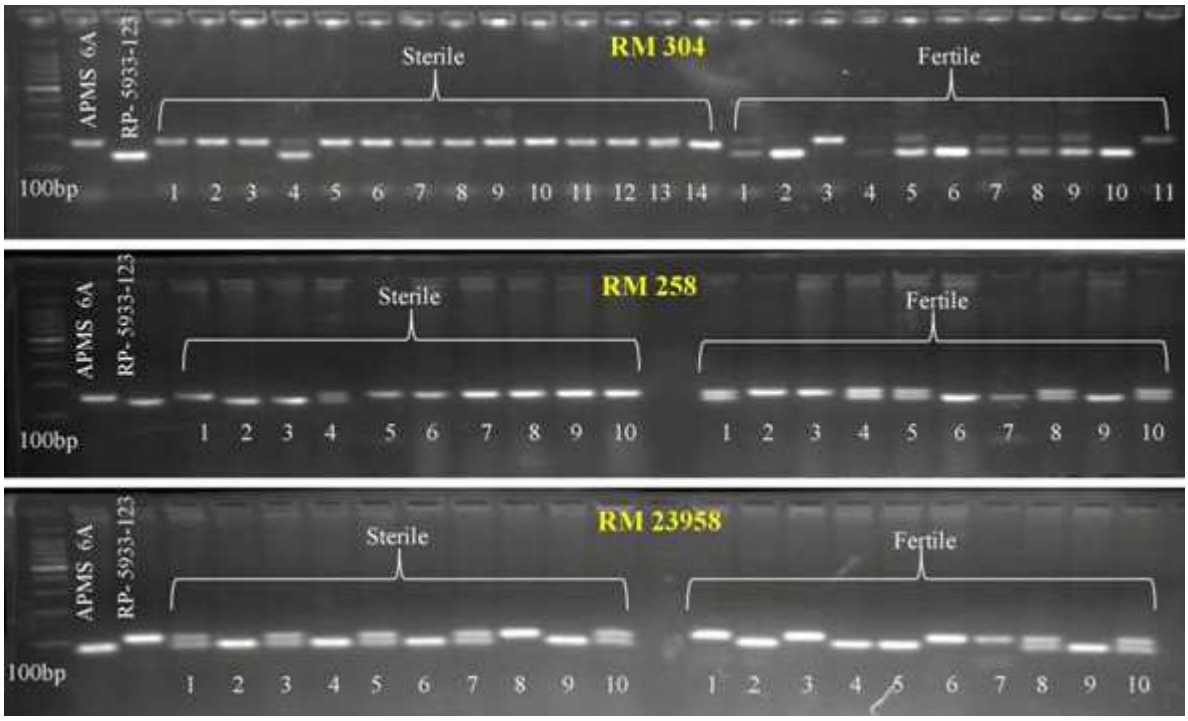

Fig. 1. Bulk segregant analysis results of SSR markers RM 304, RM 258 located on chromosome 10 and RM 23958 located on chromosome 9

Table 1. Identified polymorphic markers between parents, APMS-6A and RP-5933-123

Chromosome number	No. of markers used for polymorphism	Identified No. of polymorphic markers	Polymorphic SSR markers
1	58	8	RM495, RM3233, RM10694, RM8094, RM5853, RM10793, DRRM RF3-5, DRRM RF3-10
2	43	5	RM327, RM71, RM262, RM208, RM7485
3	40	3	RM3202, RM15971, RM6987
4	28	3	RM335, RM551, RM17604
5	32	2	RM413, RM274
6	22	2	RM19288, RM1369
7	37	5	RM125, RM20823, RM20882, RM3456, RM21328
8	27	2	RM547, RM210
9	26	1	RM23958
10	30	5	RM216, RM258, RM304, RM1108, DRCG RF4-10
11	34	2	RM287, RM206
12	36	2	RM28157, RM236
Total markers	414	40	

Most of the markers showed a complete heterozygous pattern of amplification between sterile and fertile bulks *viz*., RM10694, RM8094, RM10793, DRRM-RF_3-5, RM71, RM7485, RM335, RM413, RM1369, RM210, RM216 and RM206. The presence of both the alleles of parents in the heterozygous state is due to the chance of recombination in the segregating population (Sattari et al. 2007). Out of 40

Table 2. Chi-square analysis in the F_2 population for pollen fertility

Pollen fertility	Obs. value	Exp. value	$\chi 2$ (9:3:3:1)	P value
Fertile (>60%)	128	140.6	1.12	0.77
Partial fertile (30-60%)	52	46.9	0.55	0.90
Partial sterile (1-30%)	48	46.9	0.02	0.99
Sterile (0-1%)	22	15.6	2.6	0.46
	250	250	4.29	

Obs. = Observed; Exp. = Expected

Table 3. Chi-square analysis in the F_2 population for spikelet fertility

Pollen fertility	Obs. value	Exp. value	$\chi 2$ (9:3:3:1)	P value
Fertile (>75%)	124	140.6	1.95	0.58
Partial fertile (50-75%)	56	46.9	1.77	0.62
Partial sterile (1-50%)	51	46.9	0.36	0.94
Sterile (0%)	19	15.6	0.74	0.86
	250	250	4.82	

Obs. = Observed; Exp. = Expected

Table 4. Linkage analysis of the fertility restorer *Rf* gene by recessive class analysis

Rf gene linked marker	Segregation model[a] Chr. No.	A-B-	A-bb	aaB-	aabb	Total	$\chi 2$[b] segregation	Recom. frequency % (r-)	Map distance (cM)
*Rf*4-RM304	10	NT	NT	1	21	22	58.24***	2.27	2.27
*Rf*4-RM258	10	NT	NT	6	16	22	26.72***	13.6	13.95
RM 23958	9	NT	NT	12	10	22	4.909	27.27	31.21

a A- or B- = Fertile; aa or bb = sterile; NT = not tested.

b Marker segregation; *** indicates significance at $P = 0.01$ level for ÷2.

cM-Centimorgans (cM).

polymorphic markers, 25 showed monomorphic amplification pattern in the fertile and sterile bulks. Similar results were also obtained by Ahmadikhah et al. (2007) who explained that these markers may be located at more than 20 to 50 cM distance from the corresponding *Rf* genes.

The identified candidate markers linked to *Rf* genes by BSA were used for linkage analysis in the mapping population. Candidate markers were confirmed by recessive-class analysis (RCA) with each individual of the bulks consisting of 22 extremely sterile F_2 plants. The RCA is performed to validate and to locate the gene on its chromosome with higher efficiency (Zhang et al. 1994; Lin et al. 1996; Miyamoto et al. 1996). In the present analysis, three markers were positioned on the chromosome 10 (RM304, RM 258) and 9 (RM23958), respectively. Among the candidate marker identified through BSA, only RM 304 was verified to have a linkage for the *Rf* genes with a recombination frequency of 2.27% through RCA (Table 4). The results showed that most of the recombinant plants identified at the RM23958 loci and comparable to RM258 and RM304. The marker RM304 and RM258 were linked to the *Rf4* gene with a genetic distance of 2.27 cM and 13.95 cM, respectively on chromosome 10. RM23958 is linked to *Rf* gene with genetic distance of 31.21 cM on chromosome 9 by RCA.

The identified molecular markers associated with fertility restoration trait *viz*., RM304, RM258, and RM23598 were utilized to screen 100 lines including 98 F_2 lines along with two popular restorers *viz*., KMR 3R and RPHR 1005 to determine the efficiency of markers in identifying restorers. Based on the results it was observed that RM304 showed 90% efficiency followed by RM258 (82%) and RM23598 (45%) in identifying restorers having pollen and spikelet fertility of more than 85%. These results suggest that *Rf* gene(s) which is located on chromosome 10 might play a major role in fertility restoration whereas *Rf* gene which is located on chromosome 9 may be considered to be a minor one. The present study also confirms the previous reports that the fertility restorer *Rf4* gene which is located on chromosome 10 has major influence on fertility restoration trait compared to *Rf* genes located on different chromosomes other than chromosome 10 (Yao et al. 1997; Sattari et al. 2008, Pranathi et al. 2016 and Ponnuswamy et al. 2020).

In the present study, a new molecular marker RM23958 located on chromosome 9 and previously reported to be associated with fertility restoration trait *viz*., RM304 and RM258 located on chromosome 10 have been identified using F_2 mapping population derived from the cross APMS 6A x RP 5933-123 by bulk segregant analysis. For further confirmation and validation of these markers in governing fertility restoration of WA-CMS, it is necessary to develop isogenic lines for *Rf4* and new *Rf* locus followed by developing segregating population utilizing those isogenic lines (Hossain et al. 2010). The process of developing RILs and NILs for APMS 6A x RP 5933-123 population for further fine mapping of *Rf* genes in the newly derived restorer line in order to identify closely linked or gene based markers for utilizing them in marker assisted selections for the fertility restoration trait and restorer breeding program is under progress.

Authors' contribution

Conceptualization of research (RP); Designing of the experiments (RP, AKS, VJST); Contribution of experimental materials (RP, AKS, VJSI); Execution of field/lab experiments and data collection (VJS, AKS); Analysis of data and interpretation (VJS, RP, VGS); Preparation of manuscript (VJST, RP, AKS, DSC, VGS).

Declaration

The authors declare no conflict of interest.

Acknowledgement

Authors express their gratitude to ICAR-Indian Institute of Rice Research, Hyderabad for providing required facilities for conducting research program. The corresponding author acknowledges her sincere gratitude to Department of Biotechnology (DBT), Government of India-Biocare scheme (BT/Bio-Care/02/268/2010-11) for funding support.

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